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# Effect of Artificial Nerve Conduit Vascularization on Peripheral Nerve in a Necrotic Bed

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**Background:** Several types of artificial nerve conduit have been used for bridging peripheral nerve gaps as an alternative to autologous nerves. However, their efficacy in repairing nerve injuries accompanied by surrounding tissue damage remains unclear. We fabricated a novel nerve conduit vascularized by superficial inferior epigastric (SIE) vessels and evaluated whether it could promote axonal regeneration in a necrotic bed.
**Methods:** A 15-mm nerve conduit was implanted beneath the SIE vessels in the groin of a rat to supply it with blood vessels 2 weeks before nerve reconstruction. We removed a 13-mm segment of the sciatic nerve and then pressed a heated iron against the dorsal thigh muscle to produce a burn. The defects were immediately repaired with an autograft (n = 10), nerve conduit graft (n = 8), or vascularized nerve conduit graft (n = 8). Recovery of motor function was examined for 18 weeks after surgery. The regenerated nerves were electrophysiologically and histologically evaluated.
**Results:** The vascularity of the nerve conduit implanted beneath the SIE vessels was confirmed histologically 2 weeks after implantation. Between 14 and 18 weeks after surgery, motor function of the vascularized conduit group was significantly better than that of the nonvascularized conduit group. Electrophysiological and histological evaluations revealed that although the improvement did not reach the level of reinnervation achieved by an autograft, the vascularized nerve conduit improved axonal regeneration more than did the conduit alone.
**Conclusion:** Vascularization of artificial nerve conduits accelerated peripheral nerve regeneration, but further research is required to improve the quality of nerve regeneration. *(Plast Reconstr Surg Glob Open 2016;4:e665; doi: 10.1097/GOX.0000000000000652; Published online 22 March 2016.)*

Autologous nerve grafting remains the gold standard for repairing peripheral nerve defects. However, this method causes several donor-site problems, such as the development of painful neuromas or permanent sensory loss. The limited amount of nerves that can be harvested from the patient's own body is a critical issue for long-distance nerve gap bridging or treating multiple nerve injuries.

Recently, several types of artificial nerve conduit have been designed and used for peripheral nerve

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gap bridging as an alternative to autologous nerve grafting.[1–9] However, the conduits do not yet surpass the level of reinnervation attainable by an autologous nerve graft.[6–9] Some investigators have emphasized the importance of the intratubular vascularity of the grafted conduit in the process of nerve regeneration. Kosaka[10] reported that nerve regeneration was promoted within a silicone tube in conjunction with arterial implantation for bridging a 5-mm sciatic nerve gap in rats. Kakinoki et al[11] also showed that the distance across which axons extend can be increased to 20 mm in a rat sciatic nerve by using a silicone rubber tube containing autogenous sural blood vessels.

In the clinical setting, peripheral nerve defects are often accompanied by damage to the surrounding tissues (eg, bone or muscle defects and vascular injuries). The efficacy of an artificial nerve conduit for repairing a nerve injury combined with surrounding tissue damage remains unclear. In this study, we evaluated whether a nerve conduit could promote axonal regeneration in a necrotic bed with damage to the surrounding tissue. Moreover, we developed a novel nerve conduit that was vascularized by rat superficial inferior epigastric (SIE) vessels and compared it with a nonvascularized nerve conduit or an autologous nerve graft in terms of capacity for peripheral nerve regeneration.

## METHODS

### Nerve Conduit

Artificial nerve tube conduits (Nerbridge, Toyobo Co., Ltd, Osaka, Japan) were purchased as in a previous study.[12] The tubular conduits are made of polyglycolic acid (PGA), which is biodegradable, and are filled with medical-grade collagen. They dissolve and are absorbed by the body in about 3 months (Toyobo 2013 Annual Report. http://www.toyobo-global.com/ir/data/annual/pdf/2013/p09-10.pdf).

### Rats

Adult male LEW rats (280–320 g) were purchased from Japan SLC Inc. (Hamamatsu, Japan). All surgical procedures were performed under inhalation anesthesia with isoflurane. The animals were maintained under standard conditions with access to rodent chow and water. All procedures were performed according to the institutional guidelines of Jichi Medical University.

### Sciatic Nerve Defect and Burn Injury of Surrounding Muscle

The right sciatic nerve was exposed by making a skin incision and totally resecting the dorsal muscle in the right thigh. A 13-mm nerve segment was removed and a heated iron was then pressed against the entire dorsal thigh muscle for 5 seconds to produce a burn.[13] During this process, the proximal and distal nerve stumps were protected with wet gauze. To evaluate the morphologic changes of the muscles after the burn injury, 20 rats that received an iron scald burn injury were killed for histological analysis at 1, 2, 3, and 4 weeks after the injury (n = 5 at each time point). The damaged muscles were sectioned transversely and stained with hematoxylin and eosin (H&E).

### Implantation of Artificial Nerve Conduit Under SIE Vessels

A 15-mm nerve conduit was implanted beneath the SIE vessels to supply it with blood vessels. Briefly, the SIE vessels were exposed by a 3-cm transverse incision at the right groin in rats. Dissection of the vessels extended from the bifurcation of the femoral vessels to the lower abdominal fat tissue. Fat tissue was dissected from the abdominal wall, and the nerve conduit was implanted between the SIE vessels and abdominal wall. (See Supplemental Digital Content 1, which demonstrates the nerve repair with a vascularized nerve conduit. A, A 15-mm nerve conduit was implanted between the SIE vessels and abdominal wall in a rat groin; B, 2 weeks after implantation; C the nerve conduit and SIE vessels were elevated; and D, passed through the slit of the adductor muscle to the dorsal thigh; E, The nerve conduit was anastomosed to both stumps of the sciatic nerve. Black arrows indicate the SIE vessels, ***http://links.lww.com/PRSGO/A173***.) The fat tissue was then sutured to the abdominal muscle with 4-0 nylon to firmly fix the nerve conduit. The wound was closed with 4-0 nylon. At 2 weeks after surgery, the nerve conduit was covered with fat and connective tissue.

### Lectin Staining and Immunohistochemistry of Vascularized Nerve Conduit

To evaluate angiogenesis in the nerve conduit implanted beneath the SIE vessels, we histologically observed the vascularization in the tube at 1, 2, and 4 weeks after conduit implantation (n = 5 at each time point). The blood vessels within the conduit were visualized by labeling them with DyLight 594-labeled *Lycopersicon esculentum* lectin (Vector Laboratories, Burlingame, Calif.), using a previously reported method.[14,15] In brief, a nerve conduit was implanted beneath the SIE vessels in 15 rats as described above. At 1, 2, and 4 weeks after surgery, DyLight 594-labeled lectin (1 mg/200 g body weight) was injected into the penile vein. After 5 minutes, fixative containing 4% paraformaldehyde in 0.1 M

phosphate-buffered saline (PBS) was perfused through the vasculature under inhalation anesthesia with isoflurane. The nerve conduit was then removed and immersed in 10% sucrose for 6 hours, and then in 20% sucrose at 4°C overnight. The samples were embedded in cryomolds, and 4-μm-thick frozen sections were cut from the specimens. The sections were stained for the endothelial cell marker CD34. For immunohistochemical analyses, sections were incubated overnight at 4°C in PBS containing primary rabbit monoclonal anti-CD34 antibody (RabMab technology; Abcam, Tokyo, Japan) at a dilution of 1:600. After 3 washes with PBS, sections were incubated at room temperature for 60 minutes in PBS containing secondary Alexa488 anti-rabbit IgG antibody (Life Technologies, Tokyo, Japan) at a dilution of 1:200. To identify the nuclei, slides were counterstained with DAPI (1:1000 dilution with PBS), mounted with VECTASHIELD Mounting Media (Vector Laboratories), and observed immediately under a fluorescence microscope (BZ-9000 Generation II; Keyence, Osaka, Japan).

**Nerve Reconstruction**

Twenty-six rats were randomly allocated into 3 groups: the autograft group (n = 10), conduit group (n = 8), and vascularized conduit group (n = 8). In the autograft group, the removed nerve, which was immersed in normal saline until the time of nerve reconstruction, was reimplanted in the opposite direction across the defect with a microsurgical epineural suture of 10-0 monofilament nylon (Fig. 1A). In the conduit group, a 15-mm nerve conduit was sutured across the defect to connect the proximal and distal nerve stumps, with both nerve stumps inserted 1 mm into the conduit (Fig. 1B). In the vascularized conduit group, the defect was repaired with a nerve conduit vascularized by the SIE vessels (Fig. 1C). The conduits, implanted beneath the SIE vessels 2 weeks before nerve reconstruction, were elevated from the right groin with the vessels (see Supplemental Digital Content 1, part C, ***http://links.lww.com/PRSGO/A173***) and passed through the slit of the adductor muscle to the dorsal thigh (see Supplemental Digital Content 1, part D, ***http://links.lww.com/PRSGO/A173***). The vascularized nerve conduit was anastomosed to both stumps of the sciatic nerve (see Supplemental Digital Content 1, part E, ***http://links.lww.com/PRSGO/A173***), and the surrounding fat tissue of the flap was sutured to the dorsal thigh muscle with 4-0 nylon (Fig. 1C). The autologous nerves and conduits were anastomosed without tension in the reconstructed area. Analgesic treatment for pain relief was not performed during the postoperative period.

**Leg Muscle Contraction Test**

To examine the recovery of motor function, isometric plantar flexion at the ankle was tested by pushing the sole until the toe touched the knee, using a digital force gauge (Nidec-Shinpo Corp., Kyoto, Japan), before surgery and at day 1 and weeks 2, 4, 6, 8, 10, 12, 14, 16, and 18 after surgery.[8,16] The ratio of the muscle contraction in the right experimental leg to that in the left unoperated leg was averaged across animals in each group.

**Electrophysiological Examination**

To study the reinnervation in the regenerated nerve, electrophysiological examinations were performed at 18 weeks after surgery by using a previously reported method.[17] Under general anesthesia, an incision was made in the skin of the right limb and the sciatic nerve was exposed. The nerve was stimulated with a cathode placed 5 mm proximal to the nerve graft or conduit (0.4 mA monophasic pulse at 1 Hz; pulse duration 1 ms). The evoked compound muscle action potential (CMAP) was recorded with a needle electrode inserted in the center of the belly of the gastrocnemius muscle. Both motor latencies and amplitudes were recorded. The sciatic nerve of the contralateral healthy limb was stimulated as a positive control.

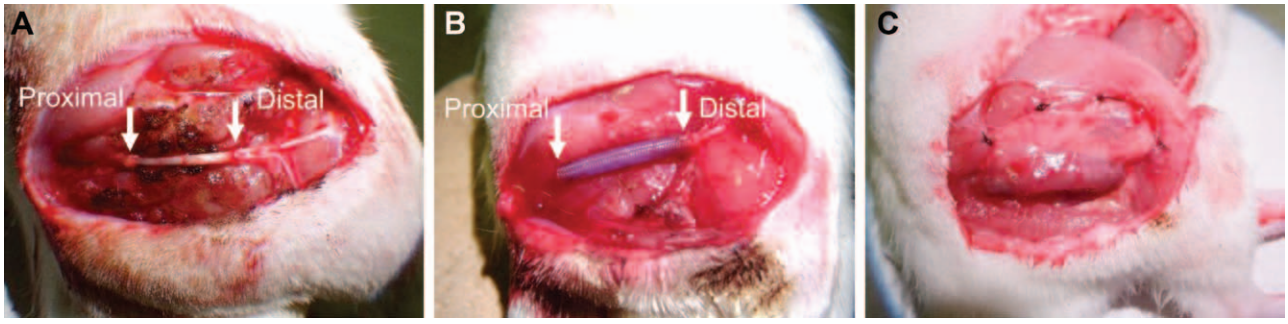


**Fig. 1.** Nerve repair with (A) autograft, (B) nerve conduit graft, or (C) vascularized nerve conduit graft. White arrows indicate proximal and distal suture sites.

### Nerve Histology

After the electrophysiological analysis, the regenerated nerves in the right experimental leg were immediately harvested from the animals. The middle and distal portions of the nerves were fixed with 10% formalin at room temperature, immersed for 2 hours in 2% osmium tetroxide (Sigma, Japan), and then embedded in paraffin. Histological specimens were observed after toluidine blue staining under a light microscope. The total number of myelinated axons for each transverse section was calculated by using ImageJ 1.48v software. For morphometric analysis of the regenerated nerves, the myelinated fiber diameter, axon diameter, and myelin sheath thickness of 100 randomly selected fibers of the distal portion of the nerves were measured. The g-ratio (the quotient of axon diameter divided by fiber diameter) was calculated to measure the degree of myelination.

### Gastrocnemius Muscle Histology and Muscle Weight Ratio

The gastrocnemius muscles from both sides were removed from the animals and weighed at 18 weeks postoperatively. The ratio of the weight of muscle from the right experimental leg to that from the left (unoperated) leg was averaged across animals in each group. Then, the middle portions of the muscles were fixed with 10% formalin and stained with H&E.

### Statistical Analysis

All results are presented as mean ± SD. Data were compared among groups by using one-way analysis of variance followed by Tukey's multiple comparison test. A $P$ value of less than 0.05 was considered significant.

## RESULTS

### Necrosis of Surrounding Muscle After Heat Damage

Morphologic changes of the damaged muscles were observed 1, 2, 3, and 4 weeks after the burn injury. At 1 week, the muscle fibers that received heat damage were necrotic as demonstrated by the lack of both nuclei and the typical sarcomere structure. Infiltration of inflammatory cells, including leukocytes and plasma cells, was observed 2 weeks after the injury. At 3 weeks, fibrous tissue was also observed, and inflammatory cells were interspersed among nonviable muscle cells. Immature muscle cells, characterized by the presence of central nuclei, appeared 4 weeks after the injury. These findings demonstrate that the burn injury created a necrotic bed, which is not an ideal soft-tissue milieu for nerve reconstruction. (See Supplemental Digital Content 2, which demonstrates the representative micrographs of H&E staining of transverse sections in the middle portions of the thigh muscle damaged by burn injury. A, 1; B, 2; C, 3; and D, 4 weeks after burn injury. Black arrows indicate nonviable muscle cells. Arrowheads indicate inflammatory cell infiltration. White arrows indicate regenerating muscle cells, ***http://links.lww.com/PRSGO/A174***.)

### Angiogenesis of Nerve Conduit

To observe angiogenesis in the nerve conduit, DyLight 594-labeled *L. esculentum* lectin was injected into the penile vein at 1, 2, and 4 weeks after conduit implantation. Fluorescence microscope observations revealed few capillary vessels in the nerve tube at 1 week but numerous capillary vessels at 2 and 4 weeks. The numbers of vessels at 2 and 4 weeks were not significantly different. [See Supplemental Digital Content 3, which demonstrates the lectin staining of nerve conduits implanted under SIE vessels. A, Representative fluorescent micrographs of the vascularized nerve conduit; B, the number of the capillary vessels in the tube at 1, 2, and 4 weeks after conduit implantation (n = 5 at each time point), $*P < 0.05$, ***http://links.lww.com/PRSGO/A175***.] On the basis of these data, we reconstructed the nerve by using the vascularized nerve conduit for 2 weeks after implanting it beneath the SIE vessels.

Two weeks after implantation, the nerve conduit was covered with fat and connective tissues, which were vascularized by the SIE vessels. The capillary vessels originating from the SIE vessels extended outside of the nerve tube. [See Supplemental Digital Content 4, which demonstrates the vascularity of an implanted nerve conduit. A, Two weeks after implantation, the nerve conduit was covered with fat and connective tissues vascularized by the SIE vessels (arrow). Asterisk indicates fat tissue. B, Capillary vessels originating from the SIE vessels extended outside of the nerve conduit (high magnification), ***http://links.lww.com/PRSGO/A176***]. Observation of longitudinal frozen sections of the nerve conduit revealed SIE vessels adhered to the outside of the PGA tube and abundant lectin-positive capillary vessels within the conduit (Fig. 2A). Fluorescence micrographs showed that the lectin staining of blood vessels closely matched CD34 immunoreactivity (Fig. 2B). These results indicate that the SIE vessels extended outside of the PGA tube and sprouted many capillary vessels into the nerve conduit to form the vascularized artificial nerve conduit.

### Functional Recovery

Recovery of motor function was evaluated before surgery and for 18 weeks after surgery. The strength of contraction of the gastrocnemius muscle gradually increased in all groups (Fig. 3). The normal contrac-

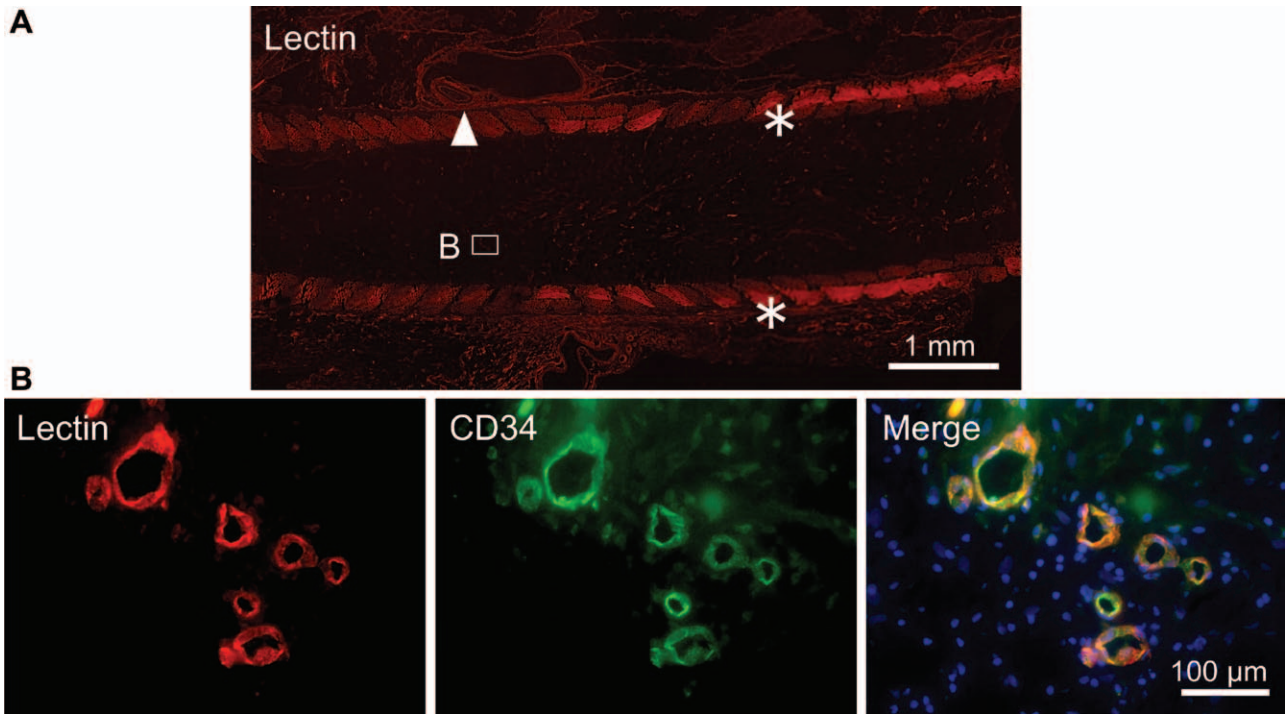


**Fig. 2.** Lectin staining of nerve conduit. A, Representative longitudinal frozen section of the nerve conduit. Asterisks indicate the walls of the PGA tube expressing red autofluorescence. SIE artery (white arrowhead) adhered to the outside of the PGA tube. B, Immunohistochemistry of the nerve conduit. Fluorescence micrographs show that lectin staining (red) of blood vessels closely matches CD34 immunoreactivity (green), and DAPI staining (blue).

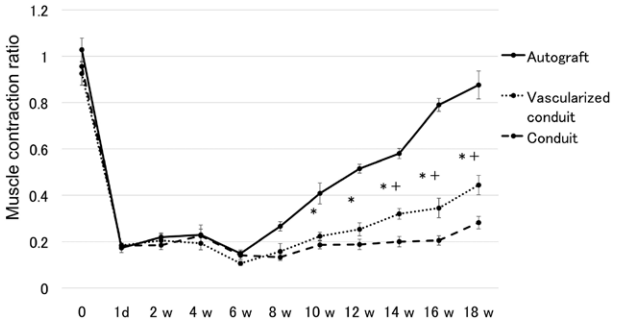


**Fig. 3.** Leg muscle contraction test. Error bars = SD. *$P < 0.05$ between vascularized conduit and autograft groups; +$P < 0.05$ between vascularized conduit and conduit groups.

tion force before injury was 0.99±0.26N. The muscle contraction forces in the autograft, conduit, and vascularized conduit groups were 0.10±0.03, 0.11±0.02, and 0.12±0.02N, respectively, at 1 day after injury and 0.80±0.19, 0.29±0.06, and 0.45±0.11N, respectively, at 18 weeks after injury. The vascularized conduit group showed significantly better motor function than the conduit group but worse motor function than the autograft group from 14 to 18 weeks after surgery.

**Electrophysiological Assessment**

To evaluate the reinnervation of regenerated nerves, electrophysiological assessment was performed 18 weeks after surgery. Both motor latencies and amplitudes were recorded in the normal nerve, autograft, conduit, and vascularized conduit groups (Fig. 4A). Terminal latencies differed significantly among the normal nerve, autograft, and conduit groups (Fig. 4B). The latency in the vascularized conduit group was shorter than that in the conduit group but similar to that in the autograft group. Compound muscle action potential amplitudes differed significantly among the 4 groups and between each pair of groups. The amplitude in the vascularized conduit group was significantly higher than that in the conduit group but lower than that in the autograft group (Fig. 4C). These results indicate that the vascularized nerve conduit improved electrophysiological nerve function more effectively than the nonvascularized conduit but less effectively than reinnervation after an autologous nerve graft.

**Toluidine Blue Staining of Regenerated Nerves**

To evaluate axonal regeneration, the middle portions of the nerves were observed after toluidine blue staining 18 weeks after surgery. (See Supplemental Digital Content 5, which demonstrates the representative micrographs of toluidine blue staining of transverse sections in the middle portions of the nerves 18 weeks after surgery. A, Normal nerve; B, autograft; C, nerve conduit; D, vascularized nerve conduit, ***http://links.***

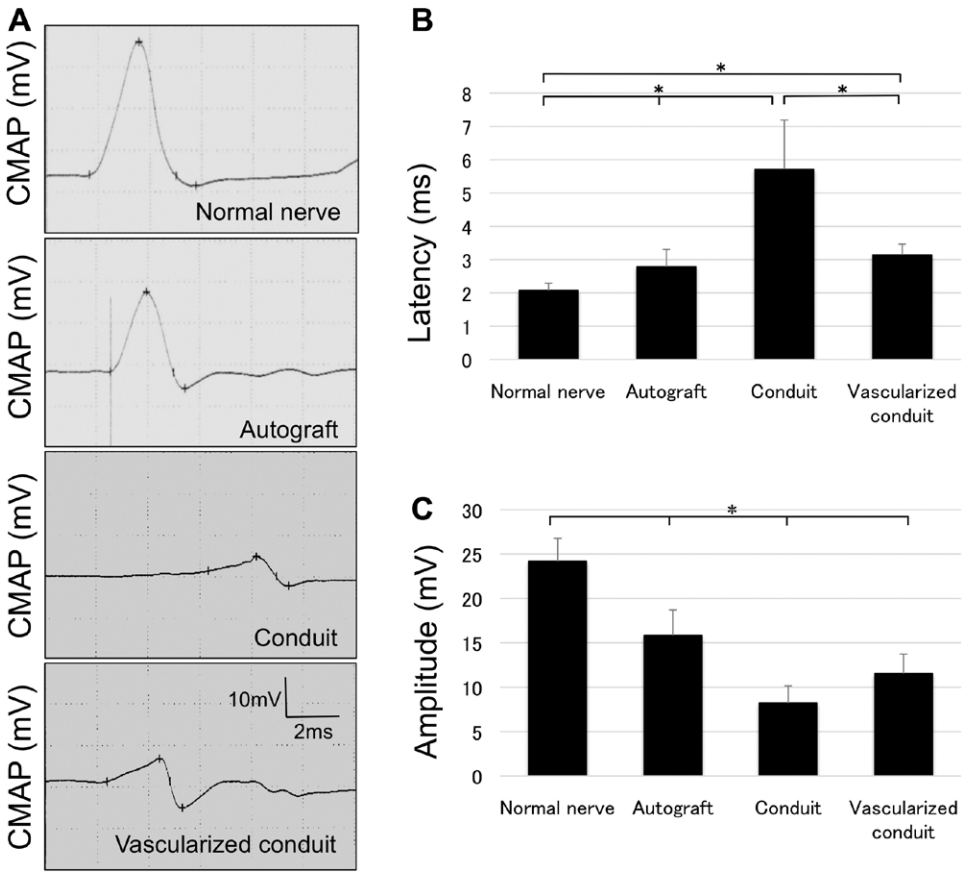


**Fig. 4.** Electrophysiological evaluation of the gastrocnemius muscle 18 weeks after surgery. A, Representative wave forms of the normal nerve, autograft, conduit, and vascularized conduit groups. One vertical division is 10 mV, and 1 horizontal division is 2 ms. B, Compound muscle action potential (CMAP) latency in the vascularized conduit group was shorter than that in the conduit group but similar to that in the autograft group. C, CMAP amplitude in the vascularized conduit group was significantly higher than that in the conduit group but lower than that in the autograft group. Error bars = SD. *$P < 0.05$.

***lww.com/PRSGO/A177***.) All groups showed myelinated axons within the regenerated nerves. The total number of myelinated axons in the conduit group was significantly less than that in either of the other groups. There was no significant difference between the vascularized conduit and autograft groups (Fig. 5).

The myelinated fiber diameters in the normal nerve, autograft, conduit, and vascularized conduit groups were 6.82±1.36, 4.47±0.06, 5.01±1.41, and 5.46±0.86 μm, respectively ($P < 0.05$ between normal sciatic nerve and other experimental groups; Figs. 6A–D). The g-ratio (the quotient of axon diameter divided by fiber diameter) in the conduit group was significantly higher than that in either of the other groups ($P < 0.05$; Fig. 6E).

### Gastrocnemius Muscle Histology and Muscle Weight Ratio

Muscles in the conduit and vascularized conduit groups were greatly degenerated at 18 weeks after surgery, whereas those in the autograft group maintained the morphology of normal muscular cells (Figs. 7A–D). The gastrocnemius muscle weight ratio

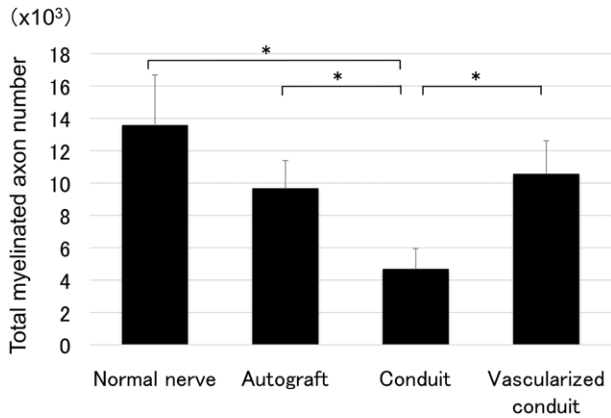


**Fig. 5.** Total number of regenerated nerves. The total number of myelinated axons in the conduit group was significantly less than that in each of the other groups. Error bars = SD. *$P < 0.05$.

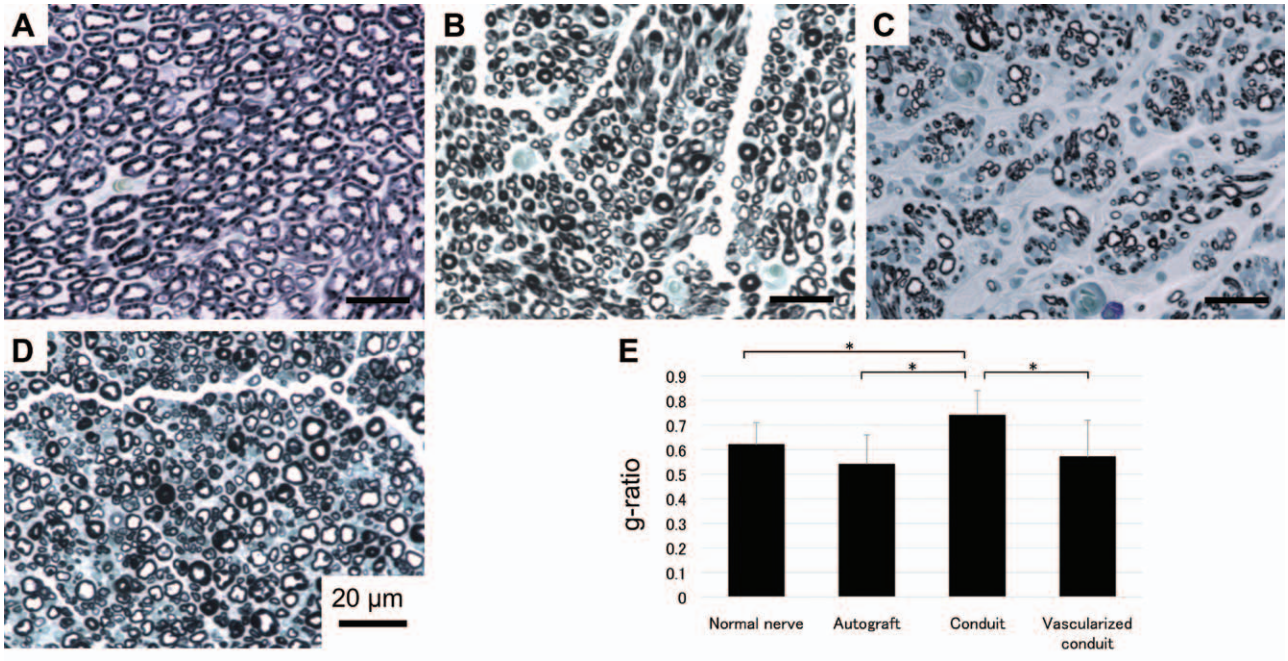


**Fig. 6.** Nerve histology and g-ratio (the quotient of axon diameter divided by fiber diameter) of myelinated nerve fibers. Representative micrographs of toluidine blue staining in the distal portions of the nerves 18 weeks after surgery in the (A) normal nerve, (B) autograft, (C) conduit, and (D) vascularized conduit groups. E, The g-ratio in the conduit group was significantly higher than that in each of the other groups. Error bars = SD. *$P < 0.05$.

showed that, whereas muscles in the autograft group were resistant to atrophy, those in both the conduit and vascularized conduit groups were not (Fig. 7E).

## DISCUSSION

In this study, rats in the vascularized conduit group showed significantly better function than

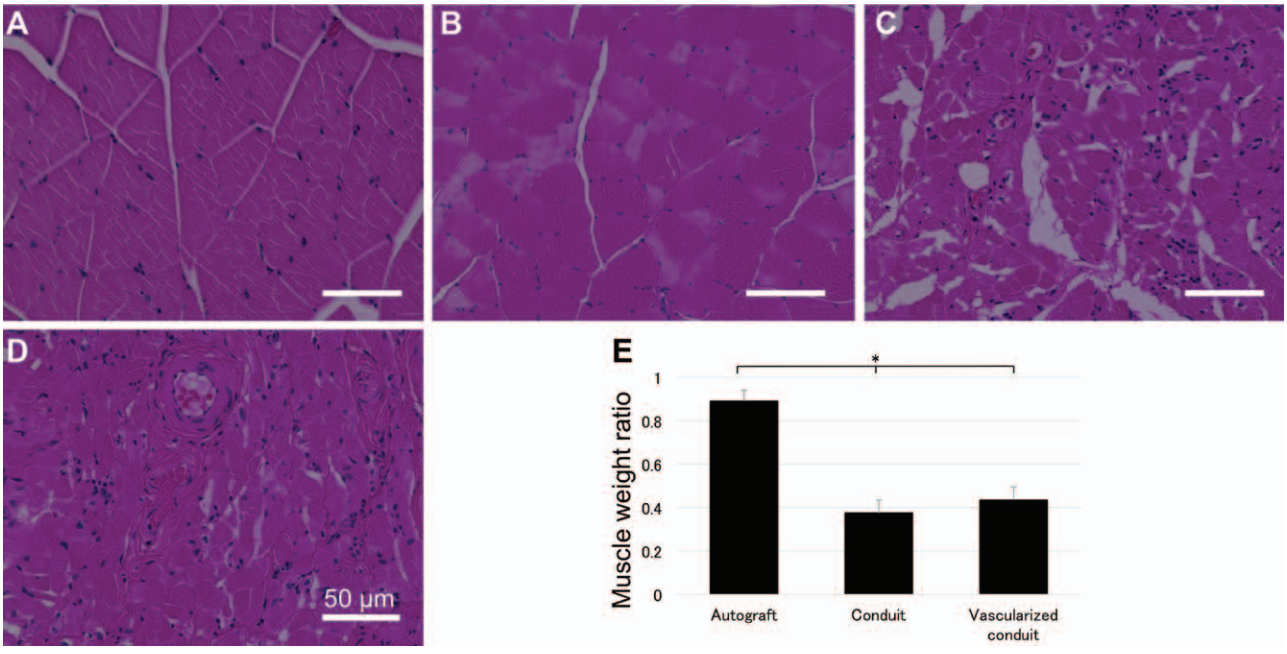


**Fig. 7.** Gastrocnemius muscle histology and muscle weight ratio. Representative micrographs of transverse sectional H&E staining of gastrocnemius muscles 18 weeks after surgery in the (A) normal nerve, (B) autograft, (C) conduit, and (D) vascularized conduit groups. E, Gastrocnemius muscle weight ratios showed that the muscles in the autograft group recovered from the atrophy, whereas those in both the conduit and vascular conduit groups greatly degenerated. Error bars = SD. *$P < 0.05$.

those in the conduit group but worse function than those in the autograft group. Electrophysiological and histological evaluations revealed that the level of reinnervation in the conduit group was significantly lower than that in the autograft group. Penkert et al[18] reported that longitudinal revascularization of the graft through the sites of anastomosis occurred within 3 days after autografting in rabbits. They also noted that revascularization from the surrounding tissue was qualitatively and quantitatively superior to that of longitudinal revascularization. Our model was achieved by a total resection of the dorsal gluteal muscle and heat injury applied to the surrounding tissue. The poor results in the conduit group indicate the importance of vascularization from the surrounding host tissue for nerve regeneration.

We designed an original nerve conduit that was vascularized by host SIE vessels and successfully fabricated a tissue-engineered conduit structure. Active bleeding from within the vascularized conduit by the SIE vessels was confirmed when the conduit was elevated from the groin. The SIE vessels extended outside of the PGA tube and formed abundant capillary vessels within the nerve conduit (Supplemental Digital Content 3 and 4, ***http://links.lww.com/PRSGO/A175*** and ***http://links.lww.com/PRSGO/A176***; Fig. 2). We suggest that the capillary vessels that originate from the SIE vessels enter both sides of the PGA tube and grow within the inner space, ultimately forming a vascularized nerve conduit. The vascularity of the nerve conduit can provide an optimal microenvironment to supply nutrients, oxygen, and several neurotrophic factors for nerve regeneration.[10,19]

In this study, vascularization of the nerve conduit facilitated the regeneration and remyelination of peripheral nerves in a necrotic bed compared with a nonvascularized nerve conduit. That the improvement of action potentials was greater in terms of latency than in terms of amplitude suggests that the major effect of the vascularity treatment is on the myelination of regenerated axons rather than on the ultimately achieved motor reinnervation. Additionally, the total number and g-ratio of regenerated nerves in the vascularized conduit group were similar to those in the autograft and normal nerve groups. Along with the electrophysiological results, comparisons of the myelinated axon count, g-ratio, and muscle weight data confirm the beneficial effects of vascularization of the nerve conduit.

A muscle contraction test, electrophysiological assessment, and histological evaluations demonstrated that the vascularized nerve conduits could not achieve the level of reinnervation attained by autografts. These results indicate that the tissue-engineered conduit can be further improved. Many investigators have attempted to implant Schwann cells in the nerve conduit because they are well known to release neurotrophic factors and support nerve regeneration in the peripheral nervous system.[20–22] By using Schwann cells, the internal milieu of the nerve conduit might be modulated for axonal regeneration. However, nonvascularized conduits filled with Schwann cells resulted in lower levels of reinnervation than autografts when they were used to bridge long nerve gaps.[20] Accordingly, we suggest that a vascularized nerve conduit combined with Schwann cells could become an optimal biocompatible nerve conduit by establishing a superior microenvironment within the tube. Further studies are needed to clarify the efficacy of combining vascularized nerve grafting with Schwann cell implantation for peripheral nerve regeneration.

## CONCLUSIONS

We demonstrated that vascularization of an artificial nerve conduit could promote axonal regeneration in a necrotic bed in which the tissue surrounding the graft was damaged. Although this tissue-engineered conduit did not achieve the level of reinnervation attained by an autograft, further studies may improve the quality of nerve regeneration beyond that of autologous nerve grafting.

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